SYNTHETIC PEPTIDE OF REGULATORY VIRUS PROTEIN R (VPR) OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) AND THE UTILIZATION THEREOF

Summary:

The invention pertains to synthetic (*s*) peptides derived from the viral regulatory protein R (Vpr) of the human immunodeficiency virus type 1 (HIV-1), particularly the chemical synthesis of the 96 amino acid full length Vpr protein, $s\text{Vpr}^{1\text{-}96}$, as well as several fragments thereof. The application of those synthetic HIV-1 Vpr peptides in biological assays, for molecular and structural characterization, as well as for the development of anti-Vpr antibodies and serodiagnostic test systems are disclosed.

So far the only *in vitro* characterized biochemical activity of HIV-1 Vpr is that of a cation-selective ion channel (Piller *et al.*, 1996, list of references at the end of examples). Those studies are based on the assumption that the C-terminal alpha-helix (amino acid position 46 to 71 in Vpr), which contains certain similarity to the honey bye poison melittin, has the capability to govern as a transmembrane anchor the formation of a membrane pore. Indeed, recombinant Vpr expressed in *Escherichia (E.) coli* was reconstituted in artificial planar lipid bilayers. Using this system, an ion channel activity was identified that is gated by the membrane potential. The gating of the channel is dependent on the positively charged C-terminal domain of Vpr which is believed to interact with the cytoplasmic part of the cell membrane.

There is evidence that Vpr forms homo oligomers: a recombinant Vpr-fusion protein was found in oligomeric structures of a molecular weight >100 kDa (Zhao *et al.*, 1994b). So far, this observation could not be confirmed by studies on viral Vpr. The molecular structure of Vpr was investigated by two laboratories using analyses of the secondary structure on short Vpr-Peptides: alpha-helical regions in Vpr positions 50-

82 was identified by NMR-studies on overlapping peptides in aqueous trifluorethanol (TFE) as well as in sodium dodecylsulfate (SDS)-micelles (Yao *et al.*, 1998). The propensity for helix formation was predicted by several authors for regions within the C-terminus as well as the N-terminus of Vpr (Mahalingam *et al.*, 1995a-d; Yao *et al.*, 1995; Wang *et al.*, 1996b). Recent studies on 25 amino acid long peptides using CD-spectroscopy in aqueous TFE solutions (Luo *et al.*, 1998) provided first experimental evidence for the existence of N- and C-terminal helices in Vpr. Based on mutational analyses, numerous and, at least partial, contradictory information was reported that tried to correlate certain primary and secondary structures of Vpr with different biological activities reported for Vpr (Mahalingam *et al.*, 1995a-d, 1997; Wang *et al.*, 1996a,b; Nie *et al.*, 1998; Di Marzio *et al.*, 1995).

The chemical total synthesis of a Vpr peptide was first described 1997 by Rocquigny and colleagues. The authors reported the synthesis of a 96 amino acid long peptide derived from the virus isolate HIV-$1_{89.6}$ (Collman *et al.* 1992). Beside the disadvantages of this synthesis reported by the authors in their publication (see in the further text) this protein is different in nine amino acid positions compared to the Vpr protein derived from the virus isolate HIV-1_{NL4-3}, the synthesis of which is described for the first time in the present specification. As such, there is a 10% difference in the amino acid sequence between the already described (Rocquigny *et al.*, 1997) and the synthetic products covering the entire as well as partial sequences of the Vpr protein derived from the virus isolate HIV-1_{NL4-3} (Adachi *et al.*, 1986) as described in detail in the present processes.

Rocquigny and colleagues (1997) did not reveal any information about the purity and the molecular characteristics of those synthetic Vpr peptides. The authors merely described far-Western blot techniques that demonstrate binding of SDS-denatured Vpr peptide with the viral nucleocapsid protein $p7^{NC}$ derived from the same HIV-1-isolate. So far, this observation of $p7^{NC}$ - Vpr - interaction could not be repeated by

any other of the numerous laboratories working worldwide in Vpr research. An important disadvantage of the Vpr synthesis described by Rocquigny and colleagues (1997) is the fact that none of the so far well-characterized biological functions of HIV-1 Vpr could be demonstrated by the authors for those synthetic peptides. Specifically, the authors show that this particular Vpr peptide does not bind to $p6^{Gag}$, a well recognized characteristic of native viral Vpr (Paxton *et al.,* 1993; Lavallee *et al.*, 1994; Kondo *et al.*, 1995; Lu *et al.*, 1995; Kondo and Göttlinger, 1996). In addition, the authors report that this Vpr peptide does not form oligomeric structures, and there are some indications that this synthetic product is insoluble in aqueous solutions. A model of Vpr - $p7^{NC}$ - interaction was introduced by the same laboratory in an additional study based on structural analyses conducted on partial sequences of Vpr peptides, however, no detailed information about structural and experimental data were provided in this or other reports published by the authors (Roques *et al.,* 1997).

Partial sequences of synthetic Vpr peptides (amino acid positions 50 to 75, 50 to 82, and 59 to 86) were used for NMR studies (Yao *et al.*, 1998). Another group applied circular dichroism spectroscopy to investigated two 25 amino acid long peptides derived from the predicted alpha helical domains in Vpr (Luo *et al.*, 1998). Furthermore, short approximately 20 amino acid long peptides derived from the C-terminal region of Vpr comprising the motif "HF/SRIG" at a concentration range of 0.7 to 3 micro-M had cytotoxic activity towards different yeast strains, for example *Saccharomyces cerevisiae*, *Candida albicans* and *Schizosaccharomyces pombe* (Macreadie *et al.*, 1996, 1997). Elevated concentrations of bivalent cations, especially magnesium and calcium, prevented uptake and thus the toxic effects of Vpr-peptides. Continuing studies provided evidence that the C-terminal Vpr peptide (amino acid positions 71-82) can induce permeabilization of membranes, the reduction of membrane potential, and eventually cell death in $CD4^+$ T cells (Macreadie *et al.*, 1997). Similar toxic effects were also for full length (Arunagiri *et al.*, 1997). For those studies the same recombinant glutathione S-transferase (GST) - Vpr - fusion

protein was used which was also employed for ion channel studies on Vpr before (Piller *et al.,* 1996). Nevertheless, as in previous studies the authors reported problems with solubility of the recombinant product in aqueous systems.

Recombinant Vpr derived from the viral isolate HIV-1_{NL4-3} was expressed in insect cells infected with recombinant baculoviruses (Levy *et al.,* 1995). The purification of those products was merely conducted by immune affinity chromatography on immobilized polyclonal antibodies directed against the N-terminal domain of Vpr. For this procedures cell culture supernatants were applied as recombinant Vpr was secreted into the culture medium.

Strategies for large scale production of recombinant Vpr have not been described thus far. In most cases, cell culture supernatants containing recombinant Vpr were used for biological assays. In such an assay it was shown that recombinant Vpr activates virus replication in PBMC (peripheral blood mononuclear cells) as well as in several monocyte and T cell lines latently infected with HIV-1. Significant disadvantages of these already described methods are:

~~- low yield that does not allow production of mg-amounts of highly purified Vpr~~ products;

- detergents were added to the recombinant Vpr during the process of affinity purification that required subsequent dialysis and renaturation;
- no studies about the potential of post translational modification of Vpr in insect cells were reported.

Expression, purification and biochemical characterization of recombinant Vpr was first described 1994 by Zhao and colleagues. For this procedure the coding sequence of Vpr protein derived from the virus isolate HIV-$1_{89.6}$ was expressed in *E. coli* as a fusion protein. For the purpose of purification of the recombinant product, the 25 amino acid FLAG epitope was fused on the C-terminus. Besides oligomerization, no

biological activity was reported for this recombinant product. A significant disadvantage of this method is the fact that Vpr is not expressed in its authentic sequence, but as a fusion protein.

In another procedure, Vpr protein derived from the virus isolate HIV-1_{HXB2} was expressed in *E. coli* as a GST - fusion protein (Piller *et al.*, 1996). After affinity chromatography on glutathione – agarose, Vpr was released from the fusion protein by proteolytic cleavage with thrombin. A significant disadvantage of the method is the fact that Vpr after thrombin cleavage tends to aggregate and could not be sustained in aqueous solution. It was reported by Arunagiri and colleagues (1997) that Vpr produced with this method could not be maintained in aqueous solution without protein precipitation and aggregation following cleavage of the GST fusion part, while only the GST - Vpr fusion protein was usable for test systems in aqueous solutions.

The patent application WO 95/26361 (Azad, A.A., Macreadie, I.G., Arunagiri, C., 1995) describes biologically active peptide fragments of HIV Vpr proteins; pharmaceutical compounds that contain those peptides or biologically active analogs thereof; antagonists of Vpr-peptides as well as pharmaceutical compounds that contain such Vpr - antagonists. The chemical synthesis of full length Vpr is not described in this method.

The patent application WO 96/07741 (Cohen, E.; Bergeron, D.; Checroune, F.; Yao, X.-J.; Pignac-Kobinger, G., 1996) protects chimeric molecules consisting of Vpr from HIV-1 and Vpx from HIV-2 that are specifically incorporated into HIV-1/HIV-2 virus particles and there interfere with the structure and function of budding virions Those chimeric molecules are protected for the application in gene therapy of HIV-1/HIV-2 infections.

The patent application WO 96/08970 (Weiner, D.B.; Levy, D.N.; Refaeli, Y., 1996) describes methods to block cell division lymphocyte activation using Vpr proteins, fragments of Vpr or sequences of vpr genes. The chemical synthesis of Vpr proteins is not described in this method.

The application of *vpr* genes in screening assay for anti - HIV - pharmaceuticals is described in US patents 5721104 and 5639619, for determination of HIV-2 infection in US patent 5580739, a Vpr-receptor -protein is described in US patent 5780238.

The invention is based on the need to develop a protocol for the high yield synthesis of Vpr proteins in mg-amounts, the purification of those Vpr proteins, and so that the end product, the highly purified Vpr proteins, can made available for general usage.

According to the invention, the problem is solved by the provision of the protein $s\text{Vpr}^{1\text{-}96}$ as well as the following peptides:

- a forty seven amino acid long N-terminal peptide ($s\text{Vpr}^{1\text{-}47}$),
- ~~a forty nine amino acid long C-terminal peptide ($s\text{Vpr}^{48\text{-}96}$)~~ and fragments of those peptides thereof, for example:
- overlapping approximately fifteen amino acid long peptides for the purpose of epitope mapping and isoelectric focusing;
- approximately twenty amino acid long peptides for the structural and functional characterization of individual domains in Vpr, particularly the peptides $s\text{Vpr}^{1\text{-}20}$ and $s\text{Vpr}^{21\text{-}40}$:

~~$s\text{Vpr}^{1\text{-}96}$:~~

~~H - Met - Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Pro - Arg - Ile - Trp - Leu - His - Asn - Leu - Gly - Gln~~

Sub. B1

- His - Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser-OH

*s*Vpr$^{1-47}$:

H-Met - Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Pro - Arg - Ile - Trp - Leu - His - Asn - Leu - Gly - Gln - His - Ile - Tyr-NH_2

*s*Vpr$^{48-96}$:

Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser-OH

*s*Vpr$^{1-20}$ as mutant *s*Vpr$^{1-20}$(Asn^{5,10,14}):

H-Met - Glu - Gln - Ala - Asn - Glu - Asp - Gln - Gly - Asn - Gln - Arg - Glu - Asn - Tyr - Asn - Glu - Trp - Thr - Leu-NH_2, and

*s*Vpr$^{21-40}$ as mutant *s*Vpr$^{21-40}$(Asn^{35}):

H-Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Asn - Arg - Ile - Trp - Leu - His-NH_2 ,

fragments of those peptides comprising approximately fifteen amino acid long peptides,

Sub. B6

sVpr$^{11-25}$:

H-Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu-NH_2,

Sub. B7

sVpr$^{41-55}$:

H-Asn - Leu - Gly - Gln - His - Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala-NH_2,

Sub. B8

sVpr$^{46-60}$:

H-Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile-NH_2,

Sub. B9

sVpr$^{56-70}$:

H-Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile-NH_2,

Sub. B10

sVpr$^{66-80}$:

H-Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg-NH_2,

Sub. B11

sVpr$^{76-96}$:

H-Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser-OH.

The C-terminal Vpr-peptide was synthesized on a serine resin using a Perkin - Elmer - peptide synthesizer. All N-terminal peptides were synthesized on a polystyrene - polyoxyethylen -resin. The chain elongation was performed using the FMOC (Fluormethyloxycarbonyl)-strategy using certain protection groups. At the end of the synthesis the cleavage of the protection groups was performed using a cleavage

solutions consisting of 95% trifluoro acetic acid (TFA), 3% triisopropylsilane and 2 to 5 % ethanedithiol, depending on the peptide length synthesized. The resin was removed, the reaction mixture was concentrated and heptane was added. Following concentration the remaining oil was digested with diethyl ether. The raw peptide was removed and lyophilized in acetic acid. Purification of the raw peptide was performed on a preparative HPLC - system (High Pressure Liquid Chromatography). All peptides were purified on a column of silica gel using a linear gradient of TFA and water in acetonitrile. The eluated peptides were concentrated and lyophilized.

Surprisingly, it was found that in contrast to previously described recombinant and synthetic Vpr products, even at mM concentration *s*Vpr - peptides are very soluble in water and remain stable without any sign of protein aggregation and protein precipitation following the described purification protocol in accordance with the invention.

It was demonstrated that the peptide *s*Vpr$^{1-96}$ adopts a folded structure, is immunologically reactive and possess biological activities comparable to native viral Vpr.

For the first time the chemical synthesis of Vpr - proteins and fragments thereof are described which comprise amino acid sequence of the virus isolate HIV-1_{NL4-3}. The term synthetic (*s*) Vpr - peptides within the scope of the present specification illustrates those peptides synthesized by solid phase peptide synthesis which comprises the authentic amino acid sequence of the native Vpr-Proteins encoded by the *vpr* gene derived from the molecular virus isolates HIV-1_{NL4-3}.

The essence of the invention lies within the combination of already know characteristics (starting materials, synthesis resin, peptide synthesizer) and novel solutions, the first chemical synthesis of those compounds, the synthesis strategy, the

specific protection groups, the cleavage resin trifluoroacetic acid-triisopropylsilane-ethanedithiole according to the invention, the application of certain gradients of solvents (TFA - water : TFA - acetonitrile) for the purpose of purification of peptides, - which are mutually influential and result in their entire action in an advantage of use and the desired success, in that synthetic sVpr-peptides are now available.

The according to the invention synthesized peptides are characterized by the following uniqueness:

They are extremely well soluble in aqueous systems enabling for the first time peptide concentrations as high as in the mM concentration range. This in turn is an essential prerequisite for following structural analysis using NMR (nuclear magnetic resonance)-spectroscopy and X-ray crystallography.

The peptides can be produced in mg amounts under economically reasonable conditions and can be purified to the highest standard. The biological characteristics and immunological reactivity of the peptides are identical to that of the native viral Vpr proteins. The peptides can be used for a variety of applications in the basic research as well as in the applied research in areas of HIV virology.

The peptides according to the invention are used in biological assays, in structural analyses of Vpr and domains thereof, for the generation of antibodies directed against HIV peptide sequences, in anti-viral reagents, for the generation of test systems for the screening of potential Vpr inhibitors, for establishment of cell culture and animal models, for the investigation of mechanisms of Vpr in HIV pathology, for *in vitro* assembly of HIV, for generation of novel vectors in gene therapy, and for the development of serological assays, specifically a Vpr capture ELISA (enzyme linked immune solvent assay).

The created products according to the invention can be used for the determination of the molecular structure of Vpr using NMR- and CD spectroscopy as well as X-ray crystallography. Those structural information in turn are essential for understanding the molecular mechanisms of Vpr proteins in the HIV replication cycle and their role in pathological mechanisms involved in AIDS related diseases. Furthermore, those products can be used for the development and the design of high throughput *in vitro* test systems to search for potential Vpr inhibitors as well as for the generation and characterization of Vpr specific antibodies and serological test systems.

The invention will be used in areas like peptide chemistry, basic research in virology, structural analyses, and medical diagnosis. The invention can be used for the generation of poly- and monoclonal Vpr specific antibodies, specifically for the generation of epitope different Vpr specific antibodies. Further areas of application are: serological test systems, specifically Vpr antigen (Ag) ELISA, as standard antigen for calibration of Vpr - Ag ELISA -techniques, for detection and quantitation of viral Vpr in blood samples of HIV infected individuals, for test systems that characterize Vpr inhibitors, for complementation of the function of endogenous viral Vpr in cultured cells infected with *vpr*-deficient HIV-mutants, for complementation of the function of viral Vpr in cultures of human lymphocytes infected with *vpr*-deficient HIV-mutants and for complementation of the function of viral Vpr in cultures of differentiated human monocytes / macrophages infected with *vpr*-deficient HIV-mutants.

The invention can be used for the characterization of reagents that :

a) block the interaction of Vpr with cellular factors, like for the glucocorticoid-receptor, transcription factors and other DNA interacting enzymes and factors;

b) regulate or block the transcription-activating function of Vpr and the activity of Vpr on steroid hormones;

c) regulate or block the transport of Vpr alone, or in conjunction with components of the HIV-pre-integration complex, and the incorporation of Vpr into budding virions during virus assembly;

d) regulate or block the Vpr-induced cell cycle arrest, and the effect of Vpr on cell differentiation and cell growth;

e) regulate or block the cytotoxic effect of Vpr, and

f) regulate or block the ion channel activity of Vpr.

Furthermore, the invention allows the application in the development and design of *in vivo* test systems for the characterization of Vpr inhibitors and animal studies.

Another advantage is that with this invention for the first time concentrated solution of Vpr can be generated for molecular, structural and function analyses necessary for the design of Vpr specific inhibitors. Another application of the invention is the reduction of the flexibility of Vpr's N-terminus using structure stabilizing factors like the UBA2-domain of the DNA repairing enzyme HHR23A which binds to Vpr, Fab-fragments derived from Vpr-specific immune globulins or viral factors, specifically components of the HIV-1 Gag polypeptide precursor $Pr55^{Gag}$ which interact with Vpr during virus assembly, the human glucocorticoid receptor or components thereof.

~~The invention support studies on the *in vitro* assembly of retroviral pre-integration~~ complex, the development of *in vitro* and/or *in vivo* applicable methods of gene transfer, DNA transfection, integration into chromosomal and episomal host DNA, or other methods of gene transfer into cells, tissues or complete organisms with the purpose of gene therapeutic application.

The following Examples serve to explain the invention, without being limited thereto.

Examples

Example 1:

Synthesis of Vpr-peptides - general protocol

Synthesis of the C-terminal Vpr-peptides was conducted using a ABI 433A synthesizer (Perkin Elmer) and a serine-resin provided by the company Fa. Rapp Polymere, Tübingen, Germany. All N-terminal Vpr peptides were synthesized on a polystyrene-polyoxyethylen-resin, "TentaGel R-RAM-resin" provided by the company Fa. Rapp Polymere, Tübingen, Germany. Synthesis of peptides was performed using the FMOC (fluoromethyloxycarbonyl)-strategy using the following protection groups: O-t.butylester for glutamate and asparagine, OtBu-ether for serine, tyrosine and threonine, Boc (tert-butoxycarbonyl-) for lysine and tryptophan, Trt (trityl - triphenylmethyl-) for histidine, glutamine and asparagine, and Pbf (2.2.4.6.7-pentamethyl- dihydrobenzofuran-5-sulfonyl-) for arginine. After finishing the synthesis cleavage of the protection groups was conducted using a mixture consisting of 95% trifluoracetic acid, 3% triisopropylsilane, and 2 to 5 % ethanedithiol, depending on the specific peptide sequence. The resin was removed, the reaction mixture was concentrated and heptane was added. Following concentration the remaining oil was digested with diethyl ether. The raw peptide was removed and lyophilized in 10% acetic acid.

Example 2:

Purification of peptides - general protocol:

100 mg of the raw peptide was purified by preparative HPLC using the Shimadzu LC-8 system. All peptides were purified on a column (300 x 400 mm Vydac-RP18-Säule, grain size 15 - 20 µM) containing column of silica gel. A linear gradient consisting of 1% TFA (trifluor acetic acid) in water 0,1% TFA in 80% acetonitrile was applied with a flow rate of 100 ml / min. Eluted peptides were concentrated and lyophilized.

Example 3:

$sVpr^{1-96}$

The peptide was synthesized on a TentaGel S-AC-resin (0.20 mmol/gram) using an ABI 433 synthesizer. At the end of the synthesis procedure, FMOC-protection groups were cleaved off and the resin was washed first with dimethylformamide and methylenchloride and then dried. The peptide was removed from the resin and purified as described above.

molecular weight: calculated: 11378 found: 11381

H - Met-Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Pro - Arg - Ile - Trp - Leu - His - Asn - Leu - Gly - Gln - His - Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser – OH.

Figure 1: $sVpr^{1-96}$ - direct separation in SDS-PAGE (A);
immune precipitation prior to SDS-PAGE (B).

Figure 2: $sVpr^{1-96}$ - preparative purification of the raw - HPLC-chromatogram.

Figure 3: $sVpr^{1-96}$ - mass spectrum (% int. and molecular weight).

Example 4:

$sVpr^{1-47}$

in analogy to examples 1 to 3.

molecular weight: calculated: 5728 found: 5728.8

H - Met - Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu

- Ala - Val - Arg - His - Phe - Pro - Arg - Ile - Trp - Leu - His - Asn - Leu - Gly - Gln - His - Ile - Tyr - NH_2.

Sub. B13

Figure 4: *s*Vpr$^{1-47}$ - mass spectrum (% int. and molecular weight).

Example 5:

*s*Vpr$^{48-96}$

Sub. B14

in analogy to examples 1 to 3.

Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser - OH.

Example 6:

*s*Vpr$^{1-20}$

Sub. B15

in analogy to examples 1 to 3.

H - Met - Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - NH_2.

Figure 5: *s*Vpr$^{1-20}$ - mass spectrum (% int. and molecular weight) (%Int. 10% =111 mV[sum= 9505 mV].

Example 7:

*s*Vpr$^{1-20}$(Asn^{5,10,14})

Sub. B16

in analogy to examples 1 to 3.

H - Met - Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - NH_2.

Example 8:

Sub. B17

*s*Vpr$^{21-40}$

in analogy to examples 1 to 3.

Wildtype-sequence:

H - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Asn - Arg - Ile - Trp - Leu - His - NH_2.

Figure 6: *s*Vpr$^{21-40}$ - mass spectrum (%Int. 10% =335 mV[sum= 28541 mV]).

Example 9:

*s*Vpr$^{21-40}$(Asn^{35})

in analogy to examples 1 to 3.

H - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Asn - Arg - Ile - Trp - Leu - His - NH_2.

Example 10:

*s*Vpr$^{11-25}$:

in analogy to examples 1 to 3.

H - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu - NH_2.

Example 11:

*s*Vpr$^{41-55}$:

in analogy to examples 1 to 3.

H - Asn - Leu - Gly - Gln - His - Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - NH_2.

Example 12:

*s*Vpr$^{46-60}$:

in analogy to examples 1 to 3.

H - Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile - NH_2

Example 13:

sVpr$^{56-70}$:

in analogy to examples 1 to 3.

H - Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile - NH_2.

Example 14:

sVpr$^{66-80}$:

in analogy to examples 1 to 3.

H - Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg - NH_2.

Example 15:

sVpr$^{76-96}$

in analogy to examples 1 to 3.

H-Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser – OH.

Literature:

Adachi, A.; Gendelman, H. E.; König, S.; Folks, T.; Willey, R. L.; Rabson, A.; Martin, M.A. (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and non-human cells transfected with an infectious molecular clone. J. Virol. 59:284-291.

Arunagiri, C.; Macreadie, I; Hewish, D.; Azad, A. (1997) A C-terminal domain of HIV-1 accessory protein Vpr is involved in penetration, mitochondrial dysfunction and apoptosis of human CD4+ lymphocytes. Apoptosis 2:69-76.

Collman, J.W.; Balliet, J.W.; Greory, S.A.; Friedman, H.; Kolson, D.L; Nathanson, N.; Srinivasan, A. (1992) An infectious molecular clone of an unusual macrophage-

tropic and highly cytopathic strain of human immunodeficiency virus type 1. J. Virol. 66:5717-5721.

Di Marzio, P.; Choe, S.; Ebright, M.; Knoblauch, R.; Landau, N.R. (1995) Mutational analysis of cell cycle arrest, nuclear localization and virion packaging of human immunodeficiency virus type 1 Vpr. J.Virol. 69:7909-7916.

Kondo, E.; Göttlinger, H.G. (1996) A conserved LXXLF sequence is the major determinant in p6gag required for the incorporation of human immunodeficiency virus type 1 Vpr. J.Virol. 70:159-164.

Kondo, E.; Mammano, F.; Cohen, E.A.; Göttlinger, H.G. (1995) The p6gag domain of human immunodeficiency virus type 1 is sufficent for incorporation of Vpr into heterologous viral particles. J.Virol. 69:2759-2764.

Lavallée, C.; Yao, X.J.; Ladha, A.; Göttlinger, H.G.; Haseltine, W.A.; Cohen, E.A. (1994) Requirement of the Pr55gag precursor for incorporation of the Vpr product into human immunodeficiency virus type 1 viral particles. J. Virol. 68:1926-1934.

Levy, D.N.; Refaeli, Y.; Weiner, D.B. (1995) Extracellular Vpr protein increases cellular permissiveness to human immunodeficiency virus type 1. Proc.Natl.Acad.Sci. USA 91:10873-10877.

Lu, Y.-L.; Bennett, R.P.; Wills, J.W.; Gorelick, R.; Ratner, L. (1995) A leucine triplet repeat sequence (LXX)4 in p6gag is important for Vpr incorporation into human immunodeficiency virus type 1 particles. J. Virol. 69:6873-6879.

Luo, Z.; Butcher, D.J.; Murali, R.; Srinivasan, A.; Huang, Z. (1998) Structural studies of synthetic peptide fragments derived from the HIV-1 Vpr protein. Biochem. Biophys. Research Communications 244:732-736.

Macreadie, I.G.; Arunagiri, C.K.; Hewish, D.R.; White, J.F.; Azad, A.A. (1996) Extracellular addition of a domain of HIV-1 Vpr containing the amino acid sequence motif H(S/F)RIG causes cell membrane permeabilization and death. Mol.Microbiol. 19:1185-1192.

Macreadie, I.G.; Kirkpatrick, A.; Strike, P.M.; Azad, A.A. (1997) Cytocidal activities of HIV-1 Vpr and SAC1P peptides bioassayed in yeast. Protein and Peptide Letters 4:181-186.

Mahalingam, S.; Ayyavoo, V.; Patel, M.; Kieber-Emmons, T.; Weiner, D.B. (1997) Nuclear import, virion incorporation, and cell cycle arrest/differentiation are mediated by distinct functional domains of human immunodeficiency virus type 1 Vpr. J.Virol. 71:6339-6347.

Mahalingam, S.; Collman, R.G.; Patel, M.; Monken, C.E.; Srinivasan, A. (1995a) Functional analysis of HIV-1 Vpr: Identification of determinants essential for subcellular localization. Virol. 212:331-339.

Mahalingam, S.; Khan, S.H.; Jabbar, M.A.; Monken, C.E.; Collman, R.G.; Srinivasan, A. (1995b) Identification of residues in the N-terminal acidic domain of HIV-1 Vpr essential for virion incorporation. Virol. 207:297-302.

Mahalingam, S.; Khan, S.H.; Murali, R.; Jabbar, M.A.; Monken, C.E.; Collman, R.G.; Srinivasan, A. (1995c) Mutagenesis of the putative alpha-helical domain of the Vpr protein of human immunodeficiency virus type 1: effect on stability and virion incorporation. Proc.Natl.Acad.Sci. USA 92:3794-3798.

Mahalingam, S.; Patel, M.; Collman, R.G.; Srinivasan, A. (1995d) The carboxy-terminal domain is essential for stability and not for virion incorporation of HIV-1 Vpr into virus particles. Virol. 214:647-652.

Nie, Z.; Bergeron, D.; Subbramanian, R.A.; Yao, X.-J.; Checroune, F.; Rougeau, N.; Cohen, E.A. (1998) The putative alpha helix 2 of human immunodeficiency virus type 1 Vpr contains a determinant which is responsible for the nuclear translocalization of proviral DNA in growth-arrested cells. J.Virol. 73:4104-4115.

Paxton, W.; Connor, R.I.; Landau, N.R. (1993) Incorporation of vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. J. Virol. 67:7229-7237.

Piller, S.C.; Ewart, G.D.; Premkumar, A.; Cox, G.B.; Gage, P.W. (1996) Vpr protein of human immunodeficiency virus type 1 forms cation-selective channels in planar lipid bilayers. Proc.Natl.Acad.Sci. USA 93:111-115.

Roques, B.P.; Morellet, N.; de Rocquigny, H.; Déméné, H.; Schueler, W.; Jullian, N. (1997) Structure, biological functions and inhibition of the HIV-1 proteins Vpr and NCp7. Biochimie 79:673-680.

de Rocquigny, H.; Petitjean, P.; Tanchou, V.; Decimo, D.; Drouot, L.; Delaunay, T.; Darlix, J.-L.; Roques, B.P. (1997) The zinc fingers of HIV nucleocapsid protein NCp7 direct interactions with the viral regulatory protein Vpr. J. Biol. Chem. 272(49): 30753-30759.

Wang, B.; Ge, Y.C.; Palasanthiran, P.; Xiang, S.-H.; Ziegler, J.; Dwyer, D.E.; Randle, C.; Dowton, D.; Cunningham, A.; Saksena, N.K. (1996) Gene defects clustered at the C-terminus of the *vpr* gene of HIV-1 in long-term nonprogressing mother and child pair: *in vivo* evolution of *vpr* quasispecies in blood and plasma. Virol. 223:224-232.

Wang, L.; Mukherjee, S.; Narayan, O; Zhao, L.-J. (1996) Characterization of a leucine-zipper-like domain in Vpr protein of human immunodeficiency virus type 1. Gene 178:7-13.

Yao, S.; Azad, A.A.; Macreadie, I.G.; Norton, R.S. (1998) Helical structure of polypeptides from the C-terminal half of HIV-1 Vpr. Protein and Peptide Letters 5:127-134.

Yao, X.-J.; Subbramanian, R.A.; Rougeau, N; Boisvert, F.; Bergeron, D.; Cohen, E.A. (1995) Mutagenic analysis of human immunodeficiency virus type 1 Vpr: role of a predicted N-terminal alpha-helical structure in Vpr nuclear localization and virion incorporation. J.Virol. 69:7032-7044.

Zhao, L.J.; Mukherjee, S.; Narayan, O. (1994a) Biochemical mechanism of HIV-1 Vpr function: specific interaction with a cellular protein. J. Biol. Chem. 269:15577-15582.

Zhao, L.J.; Wang, L.; Mukherjee, S.; Narayan, O. (1994b) Biochemical mechanism of HIV-1 Vpr function: oligomerization by the N-terminal domain. J. Biol. Chem. 269:32131-32137.

Zhao, Y.; Cao, J.; O'Gorman, M.R.; Yu, M.; Yogev, R. (1996) Effect of human immunodeficiency virus type 1 protein R (vpr) gene expression on basic cellular function of fission yeast *Schizosaccharomyces pombe*. J. Virol. 70:5821-5826.

Figure legends:

Figure 1: Structural and functional domains in Vpr

The following primary and secondary structural motifs are aligned to the amino acid sequence of the Vpr protein derived from the Isolate HIV-1_{NL4-3}: The negatively charged N-terminus (label (1), positions 1 - 17); helix alpha-1 (Label (2), positions 18 - 37); a not further defined region (label (3), positions 38-51); helix alpha-2 (label (4), positions 51 - 76); a positively charged C-terminus (label (8), positions 77 - 96). Overlapping to labels (1) to (5) and (87) the following domains are indicated: a leucine- and isoleucine-rich regions termed as "leucine - zipper - like or "LR-domain" (label (5), positions 60 - 80); a region containing the repetitive motif "HF/SRIG" (label (6), positions 71 - 82); the predicted transmembrane anchor of Vpr required for the ion channel activity of Vpr (label (7), positions 52-79).

Figure 2: Immunological characterization of polyclonal antibodies specific for sVpr$^{1-96}$ by Western blot and immune precipitation.

Rabbits were immunized with sVpr$^{1-96}$ and the resulting serum *R-96* was tested in Western blot (A) and immune precipitation (B). A serial dilution of sVpr$^{1-96}$, 0.01 to 10 ng, was separated in a SDS-PAGE (12.5% acryl aide gel) (A). A similar serial dilution of sVpr$^{1-96}$ was added to human serum and from the mixture sVpr$^{1-96}$ was recovered by immune precipitation using the serum *R-96* followed by separation of the immune precipitates in a SDS-PAGE (B). sVpr$^{1-96}$ was electro-transferred onto PVDF-membranes and the peptide was detected using *R-96* followed by binding to

^{125}I- labeled protein G. The autoradiogram of a two-day exposure is shown in (A) and (B). Positions of the molecular weight standard proteins are indicated on the left, positions of the light (lc) and heavy chain (hc) of immune globulins used for immune precipitation are indicated on the right.

Figure 3: *s*Vpr$^{1-96}$ activates virus replication and increases the number of live cells in cultures of human PBMC.

Cultures of PHA- and IL-2-activated PBMCs were infected with equal infectious doses of the following virus stocks: HIV-1_{NL4-3} (A, B, C), NL4-3(AD8) (D) as well as the *vpu*-deficient mutant NL(AD8)-U_{DEL1} (E), and the *vpr*-deficient mutant NL(AD8)deltaR (F). During the course of the experiment cultures were incubated with 10 nM of *s*Vpr$^{1-96}$ or 10 nM of the control peptide Vpu$^{32-81}$. Virus release is demonstrated as the profile of virus associated RT-activity released into the cell culture supernatant (A,C,D,E,F). (B) shows the number of live cells detected in the cultures of experiment (A).

Figure 4: *s*Vpr$^{1-96}$ activates virus replication of *vpr*-deficient HIV-1 mutant viruses in cultures of primary human monocytes/macrophages isolated from different donors. Parallel cultures of differentiated MDM isolated from three different donors were infected with equal infectious doses of purified virus stocks of the macrophage-tropic virus NL4-3(AD8) as well as the *vpr*-deficient mutant NL(AD8)deltaR. Virus production was followed over a time frame of two months and release of virus associated RT-activity was plotted against time.

Figure 5: 2D ^{1}H TOCSY spectrum.
Mixing time was 110 ms, the spectrum was recorded of a 2 mM solution of *s*Vpr$^{1-96}$ in 1:1 ($^{V}/_{V}$) TFE-d2/H2 at 300°K. The x- and y- axes demonstrating the respective 1D ^{1}H spectra. Enlargements of regions A, B and C are shown in figure 6.

Figure 6: Enlargements of regions of a 2D TOCSY spectrum shown in figure 5 that correlates to interaction of protons between proline H-7 and H-2 of tryptophan residues (A); H-2 and H-4 of histidine residues (B), and epsilon-H and alpha-H of arginine residues (C).

Figure 7: *s*Vpr$^{1\text{-}96}$ - HPLC chromatogram and mass spectrum.

Figure 8: *s*Vpr$^{1\text{-}47}$ - mass spectrum.

Figure 9: *s*Vpr$^{1\text{-}20}$ - mass spectrum.

Figure 10: *s*Vpr$^{21\text{-}40}$ - mass spectrum.